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Cuticular lipids and silverleaf whitefly stage affect conidial germination of *Beauveria bassiana* and *Paecilomyces fumosoroseus*

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Abstract

Beauveria bassiana and *Paecilomyces fumosoroseus* are generalist entomopathogenic fungi that infect the silverleaf whitefly (*Bemisia argentifolii*). We found second and third instar whiteflies to be the most susceptible larval stage to both fungi. Conidia of *B. bassiana* germinated most readily on the cuticle of second instars (54% germinated) and *P. fumosoroseus* germination was highest on third instar cuticle (45%). Fourth instars (the ultimate instar) had low susceptibility to these pathogens, and spore germination on the cuticle of fourth instars was very low for *B. bassiana* (7%) and intermediate for *P. fumosoroseus* (33%). Cuticular lipids were found to have toxic or inhibitory effects on conidia of *B. bassiana* and *P. fumosoroseus* when the spores were germinated on nutrient agar in the presence of the lipids. In the absence of added nutrients, *P. fumosoroseus* conidial germination increased in the presence of the lipids. To test if the inhibitory effects of the lipids were due solely to hydrophobicity (preventing water from coming into contact with the conidia) we tested the effects of synthetic long-chain wax esters. The synthetic wax esters inhibited germination of *P. fumosoroseus* to a degree that was similar to the effect of the cuticular lipid extracts, but the synthetic lipids did not have a significant effect on *B. bassiana*. Thus, the thick coating of long-chain wax esters produced by whitefly nymphs affect spore germination of fungal pathogens, but whether they play a significant role in defense against disease is not clear.
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Keywords: Wax esters; *Bemisia*; Whiteflies; Spore germination; *Beauveria*; *Paecilomyces*; Insect pathogens; Entomopathogenic fungi; Cuticular lipids

1. Introduction

Paecilomyces fumosoroseus and *Beauveria bassiana* are mitosporic fungi that are pathogenic to a wide variety of insects. These fungi are easily cultured in vitro, and thus lend themselves for use as microbial pesticides for several arthropod pests, including the silverleaf whitefly, *Bemisia argentifolii* (Faria and Wraight, 2001). If *B. bassiana* conidia are germinated prior to being applied to whitefly nymphs, the infection rate is higher than when ungerminated conidia are used (James, 2001). One possible explanation for this result is that the cuticle is unfavorable to spore germination, by either lacking the proper stimuli for, or by directly reducing or inhibiting germination.

Silverleaf whitefly nymphs are covered with a very thick layer of cuticular lipids, mainly long-chain (C_{42}–C_{64}) wax esters (Buckner et al., 1999). These copious amounts of wax could potentially affect spore germination by fungilytic or fungistatic toxicity, or by acting as a barrier to the chitin matrix of the insect exoskeleton, effectively preventing the spore from coming into contact with nutrients or other cues that trigger germination. Medium- and short-chain fatty acids and alcohols have been demonstrated to have toxicity to filamentous fungi (Pedersen, 1970; Rolinson, 1954; Teh, 1974), including some that have been isolated specifically from insect cuticle (Koidsumi, 1957; Saito and Aoki, 1983; Smith and Grula, 1982), but the toxicity of long-chain fatty acids is unknown. We used scanning electron microscopy to determine whether spore germination of

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both *B. bassiana* and *P. fumosoroseus* was inhibited on nymphal cuticle, testing different nymphal instars, and then extracting cuticular lipids to determine whether they had an inhibitory effect on conidia.

2. Materials and methods

2.1. Sources of fungi

Paecilomyces fumosoroseus 612, was obtained from Mark Jackson (National Center for Agricultural Utilization Research, USDA-ARS, Peoria, IL). It was originally isolated from *B. argentifolii*. *B. bassiana* GHA was obtained from Mycotech Corporation (Butte, MT). It is the active ingredient in Mycotrol (Mycotech, Butte, MT). To assure good pathogenicity, each culture was used to infect silverleaf whitefly nymphs, and was then re-isolated on Saboraud dextrose agar (Difco Laboratories, Detroit, MI) modified with 1% yeast extract (SDAY). The re-isolated cultures were maintained on SDAY at 25 °C for the duration of the experiment, but were never cultured more than four cycles past the original re-isolate from whitefly nymphs. For each experiment, the fungi were always grown on SDAY for 14 days at 25 °C with a 10:14 h light:dark photoperiod. Petri plates were sealed with parafilm for 1 week, after which time the seal was removed to allow the plates to dry out and stimulate the production of conidial spores. Freshly collected conidia from 14-day-old cultures were used for every experiment and each replicate run.

2.2. Fungal pathogenicity to different stages of the silverleaf whitefly

Dose–response tests were conducted against second, third, and fourth instar nymphs. For each instar, we tested five concentrations of *B. bassiana* and *P. fumosoroseus* (2×10^8, 6.6×10^7, 2.2×10^7, 7.4×10^6, and 2.5×10^6 conidia per ml), and a control. A haemocytometer was used to enumerate the highest concentration, and a series of threefold dilutions was used to obtain the other concentrations. The conidia were mixed using a vortex mixer and a surfactant (0.01% Silwet L-77, Loveland Industries, Greeley, CO) to obtain uniform suspensions, and the control consisted of 0.01% Silwet L-77. Because it is difficult to test all instars at the same time, and date effects are common with bioassays, each instar was tested independently, three different times, and in random order.

For these bioassays, the silverleaf whitefly was maintained on cantaloupe, eggplant, and hibiscus plants in a walk-in growth chamber at approximately 25 °C, with a photoperiod of 14:10 h light:dark (as described by James, 2003). For each experiment, sweet potato leaves were collected from plants grown in the greenhouse, and then soaked in a weak solution of bleach and dish detergent in cold water (approximately 5% bleach and 0.3% detergent) for several minutes to kill any mites, whiteflies, fungi, and other pests that might be infesting the leaves at low levels. The leaves were then rinsed three times in cold water and placed in water piks (Aquatube, Syndicated Sales, Kokomos, IN) with nutrient solution (0.03% Peters Excel, The Scotts, Marysville, OH) overnight to dry and regain turgor.

The leaves developed roots under these conditions and remained in good condition for several weeks. The leaves were then placed in a screened cage inside the whitefly chamber described above. Whitefly adults from the colony were shaken from their host plants into the cage until enough whiteflies were present (based on previous experience) to infest the plants with at least 40 eggs per leaf when left for 48 h. After 48 h, the leaves were taken out of the cage and all the adult whiteflies were removed from the leaves by blowing them off by mouth. The leaves were incubated in another growth chamber (free of adults) at 25 °C, with a photoperiod of 14:10 h light:dark, until the nymphs had developed to the appropriate stage, which was 10 days for second instars, 14–16 days for third and fourth instars.

For each experiment, leaves infested with whiteflies were examined under a dissecting scope, and 40 nymphs were marked on each leaf by placing a small black dot near each nymph using a fine tipped permanent marker. If the nymphs were crowded together in a particular spot, making it difficult to identify which one was supposed to be marked, then a very fine insect pin was used to remove some of the other nymphs. In each experiment, three leaves and 120 insects were used per treatment (a treatment is one instar, one dosage). A total of nine experiments were set up, three for each instar (for each instar, both fungi were tested at the same time). Two of these experiments were set up at the same time (one time we used third and fourth instars co-occurring on the same leaves), but all the other experiments were set up on different dates and assigned randomly.

The day after the nymphs were marked, the leaves were taped flat on acrylic plates and sprayed on the abaxial side (where the nymphs occurred) with 1 ml of each spore concentration using a Potter Precision Laboratory Spray Tower (Burkhard Manufacturing, Rickmansworth, England) with 0.7 kg/cm^2 pressure and the fine-mist nozzle (James and Jaronski, 2000). The sprayer was calibrated to determine the delivery rate of spores. To calibrate the sprayer, three concentrations of spores were sprayed onto an agar plate, and then the number of spores per square millimeter was counted using a phase contrast microscope at 400×. The three concentrations sprayed in the calibration were 2.72×10^8, 5.44×10^7, and 1.09×10^7 spores per ml, and the calibration was replicated three times. Regression analysis was used to determine the

conversion rate [spore density (spores/mm^2) = (7.31×10^{-6})(n spores/ml)] ($P \leqslant 0.0001$, $r = 0.9993$). Using this equation, we determined that our dosage rates were 0, 91, 183, 365, 731, and 1462 spores/mm^2. The standard error of the slope parameter for the regression was 9.50×10^{-8}.

The leaves were removed from the acrylic plates, set upright in test tube racks, allowed to dry in the laboratory, and then incubated at 25 (± 2) °C, 94–98% RH (humidity was affected by the light cycle, being at 97–98% for 12 h during the dark phase, but dropping to as low as 94% during the last few hours of the light phase). The photoperiod was 14:10 h light:dark. After 24 h, the bioassay was moved to 25 (± 2) °C, 70% RH (actual RH was 70–85%). A small temperature and relative humidity recorder (Hobo ProSeries, Onset Computer, Pocasset, MA) was placed in the incubator during each replicate run to record environmental conditions. Insect mortality was recorded 7 days after each spray application. Insects were considered dead if they had turned red from *B. bassiana* infection, if they lost their transparent appearance and took on a clouded color, if fungal hyphal growth was seen protruding from the insect, or if the nymph became overly flattened and dry in appearance, curling up around part of the edge.

Spore viability was tested at the time of each replicate run by plating 0.1 ml of the middle concentration (2.2×10^7 conidia/ml) on a 9 cm petri plate with SDAY and incubating it in the same incubator as the treated insects. After 20 h of incubation, the proportion of viable conidia was determined by observing 500 conidia at 400× magnification with a phase contrast microscope and counting the number that had germinated (a spore was considered to have germinated if a distinct germ tube had formed). Spore germination rates were always $\geqslant 95\%$.

Data were analyzed with probit analysis. The dependent variable was the proportion of larvae that died, using Abbott's (1925) formula to adjust mortality based on the control mortality (control mortality in each replicate was always less than 20%). All the data were put into one probit model, using Proc Genmod (SAS, 1999), which included fungal species, instar, log dose, date, and all interactive effects (except those involving date) as fixed effects to determine which interactive effects were significant. Both the slopes and intercepts were allowed to vary in this first model. The dose by instar and dose by fungal species interactions were not significant, and so were removed from the model. The experiment was not a block design because the different instars were treated at different times. However, all the dosages and both of the fungi for a given instar were tested at once for each replicate run, and for this reason, date was included in the model (i.e., each dose response test was replicated on a different date).

Using this model, a likelihood ratio test of equality was used to test the null hypothesis that the probit lines were equal for the two fungi (compared for each instar separately). Another likelihood ratio test of equality was used to test the null hypothesis that the probit lines were equal for each pair of instars for a given fungus (compared for each fungus separately). A total of nine pair-wise comparisons were made, which is greater than the treatment degrees of freedom, so a Bonferroni adjustment of α was used to keep the experiment-wise error at 5% ($P_{\text{critical}} = 0.006$). For each fungus, the probit slopes of the different instars were not significantly different, so we removed the dose x instar interactive term, effectively forcing all the slopes to be the same for this comparison.

2.3. Conidial germination on whitefly cuticle

Whitefly nymphs were produced as previously described, except that the infestation time for the leaves was such that we had leaves with eggs, second, third, and fourth instars available all at once (but one stage per leaf). At the location where this experiment was done, we did not have a Potter tower available, so *B. bassiana* and *P. fumosoroseus* conidia, at a concentration of 2×10^7 conidia/ml (in 0.01% silwet), were placed in a Nalgene aerosol spray bottle (Nalgene Nunc International, Rochester, NY) and sprayed on the infested leaves until just wetted, followed by drying for ~1 h. The leaves and nymphs were then sealed in large, clear, plastic bags (to elevate humidity levels) and placed at 25 °C for 24 h.

After 24 h, conidial germination on the larvae was observed with a scanning electron microscope (model JSM6300, JEOL, Peabody, MA). The samples of conidia on the nymphs were examined fresh without fixation or dehydration. Pieces of leaves bearing the treated nymphs were cut from the whole leaf and sputter coated with Au/Pd using a Balzers SCD 030 sputter coater (Bal-Tec Ag, Balzers, Liechtenstein).

A total of 150 conidia were counted for each larval instar, from 3 to 6 insects. Eggs had fewer conidia on them, so nine eggs were counted for the *B. bassiana* treatment (162 conidia), and six eggs for *P. fumosoroseus* (201 conidia). We compared percent germination between each instar for each fungal species (each fungus was compared separately), and then compared the differences between the two fungi for each life stage of the whitefly. To do this, we calculated the odds ratios (the ratio of the probabilities for germination for the two treatments being compared) using logistic regression (Proc Logistic, SAS, 1999). If an odds ratio was significantly different than one, then the two treatments were considered to differ significantly. The total number of comparisons made was 18, so we used a Bonferroni adjustment on the critical P value ($\alpha = 0.05$, $P \leqslant 0.003$) for these tests.

2.4. Effect of cuticular lipids on conidial germination

To obtain cuticular lipids from *B. argentifolii* nymphs, the whiteflies were grown on hibiscus plants in cages that were placed within a walk-in environmental chamber with 15:9 h light:dark and 28:24 °C day:night. Cantaloupe plants (variety 'Hymark') were grown on peat pellets (Jiffy Products, Shippan, Canada) for 1 week before being placed within whitefly colony cages for 2–3 days. The whitefly egg-laden plants were removed from the colony cages and held in a separate environmental chamber, with the same temperature and lighting conditions, until the nymphs reached the third instar.

Cuticular lipids were removed only from third instar *B. argentifolii* nymphs (nymphs with a body length between 450 and 550 μm were considered third instars). Third instars were removed from leaves with a 0.12-nm gauge pin and transferred to 7-ml, ice-chilled glass vials. Groups of 200–300 nymphs were collected per vial. The vials were warmed to room temperature and cuticular lipids were extracted from pooled nymphs by submersion in approximately 1 ml of hexane for 1 min. The hexane was decanted and filtered through a small plug of glass wool. Hexane extracts were combined to give a single pooled sample from 3480 nymphs. After evaporation of the hexane with nitrogen gas, the lipid residue was stored under argon at −20 °C. For the experiment, the pooled extract was dissolved in 58 μl of chloroform ($CHCl_3$). A 2 μl aliquot represented a 120-nymph equivalent. Twofold serial dilutions of this initial solution were prepared to give solutions whereby 2 μl amounts represented 60-, 30-, and 15-nymph equivalents.

The lipid extracts were applied to mixed cellulose ester filter membranes (43 mm diameter, 0.8 μm pore size, gridded, Millipore, Bedford, MA), and these treated membranes were then used as a substrate for spore germination.

The membranes were cut with fine scissors into 3×3 mm squares, with each square showing grid lines on one side. The squares were cleaned three times by submersion in $CHCl_3$. For application of lipid to the membranes, fine forceps were used to hold a corner of each membrane square and the gridded side of the membrane was quickly placed onto a 2 μl drop of each lipid solution that had been placed in the bottom of a 1-ml well of a glass well plate. The $CHCl_3$ solution was immediately absorbed into the membrane square. After evaporation of the $CHCl_3$, the lipid-coated membrane squares were stored in screw-capped glass vials under argon. Nine membrane pieces were treated for each cuticular lipid dilution.

To determine whether the effect of the cuticular lipid on spore germination was a property particular to the whitefly extracts, or whether it was a general effect of long-chain wax esters, two commercially available wax esters, docosanyl eicosanoate (42 carbons) and triacontanyl tetracosanoate (54 carbons) (Nu-Chek- Prep, Elysian, MN) were used as control lipids. Neither of these synthetic wax esters are known to be produced by insects. These wax esters were dissolved in $CHCl_3$ to give an initial high concentration of 4 mg/ml (this was the concentration we estimated the cuticular lipids to be based on sample weight). Membrane squares were treated with 2 μl of that concentration, as well as, serial dilutions at 2, 1, and 0.5 mg/ml. To aid in dissolving the 54 carbon wax ester, a solvent mixture of 25% benzene in $CHCl_3$ was used.

Three membranes from each of the cuticular lipid treatments (including three membranes washed in $CHCl_3$ but not treated with lipid, as a positive control) were sprayed with either *B. bassiana* or *P. fumosoroseus* using the Potter Precision Spray Tower. Fungal conidia were mixed to a concentration of 6.5×10^7 conidia/ml on 0.01% Silwet L-77. For each fungus, all the membrane pieces were arranged in one petri dish and the dish was sprayed with 1 ml of conidial preparation. For each cuticular lipid concentration and each fungus, three membrane pieces were incubated in sterile water, and three were incubated in sterile 0.5% yeast extract. The water and yeast extract solutions were applied to the sterile cellulose membrane pads (Fisher Scientific, Pittsburgh, PA) that came with the membranes, until the pads were soaked, and then the membrane pieces were placed on the pads and incubated at 25 °C for 12 hours. The soaked pads in the petri dishes provided saturated conditions for the spores. Based on preliminary experiments, 12 h was estimated as the time needed for 90–100% germination, without allowing for hyphae to develop and overgrow some of the conidia and prevent an accurate determination of percent germination.

Spore germination on the membranes was determined using SEM. The membrane pieces with conidia were mounted on stubs, sputter coated with Au/Pd and examined fresh, unfixed. To determine percent germination, we counted the first 50 conidia found on each membrane piece and noted how many had germinated. We used three membrane pieces per treatment, for a total of 150 conidia per treatment. Logistic regression (Proc Logistic, SAS, 1999) was used to test whether fungal species, incubation medium (water versus yeast extract solution), or whitefly cuticular lipid concentration had significant effects on spore germination. The interactive terms for these main effects were included in the models, when significant.

For the experiment using the synthetic lipids, logistic regression was used to test whether lipid type, fungal species, or lipid concentration had significant effects on spore germination. The interactive terms for these main effects were included in the model.

3. Results

3.1. Fungal pathogenicity to different stages of the silverleaf whitefly

Different whitefly life stages showed significantly different susceptibilities to both *B. bassiana* and *P. fumosoroseus* (Fig. 1). The effect of test date in the probit model was significant ($X^2 = 13.29$, $P = 0.04$), as was the instar by fungal species interaction ($X^2 = 10.79$, $P = 0.005$). The latter is an indication that differences between instars was not the same for both fungi. For *B. bassiana*, second and third instar larvae were the most susceptible life stages ($X^2_{\text{2nd vs. 3rd}} = 10.20$, $X^2_{\text{2nd vs. 4th}} = 29.59$, $X^2_{\text{3rd vs. 4th}} = 19.61$, $\alpha \leqslant 0.05$) (Table 1, Fig. 1). Although the intercepts differed significantly between these second and third instars, the mortalities were clearly similar within the range of dosages tested. Third instars were the most susceptible stage to *P. fumosoroseus* infection ($X^2_{\text{2nd vs. 3rd}} = 5.54$, $X^2_{\text{4th vs. 3rd}} = 18.82$, $\alpha \leqslant 0.05$) (Fig. 1). Both fungi had fairly low pathogenicity to fourth instars (Fig. 1).

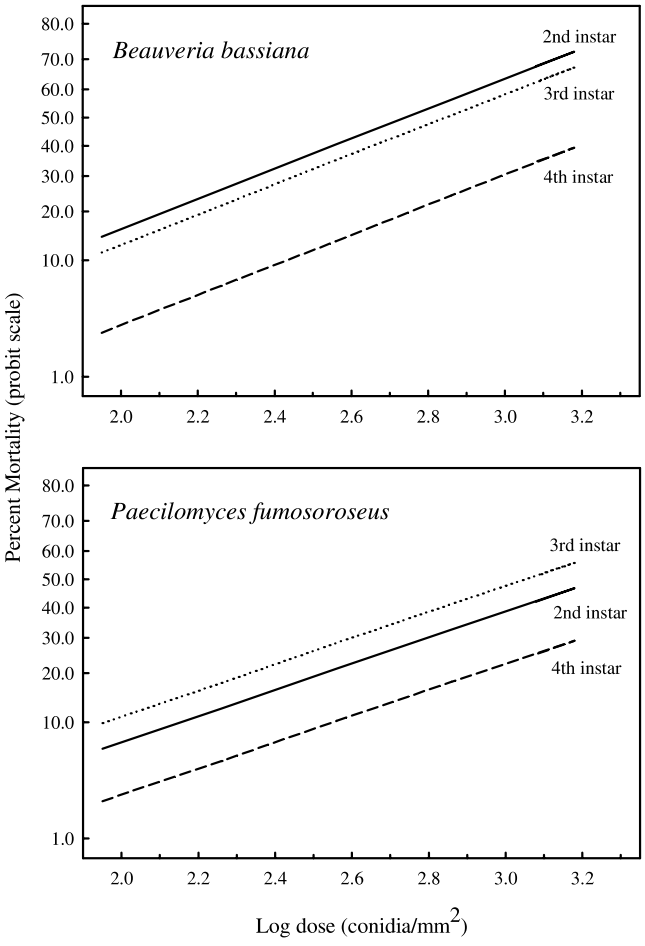

Fig. 1. Probit lines for mortality of *Bemisia argentifolii* nymphs exposed to different dosages of conidia from *Beauveria bassiana* and *Paecilomyces fumosoroseus*. Percent mortality is on a probit transformed scale.

Mean control mortality was 2.0% (SE = 1.1%) for second instars, 11.6% (1.4%) for third instars, and 9.6% (3.9%) for fourth instars.

3.2. Conidial germination on whitefly cuticle

Very few conidia germinated on eggs, for both fungi (Table 2). *B. bassiana* germinated more readily on seconds than did *P. fumosoroseus*, but *P. fumosoroseus* germinated more readily than *B. bassiana* on thirds and fourths (Table 2). In comparing the germination rates on different instars, for a given fungus, *B. bassiana* was found to germinate most readily on second instars; whereas, *P. fumosoroseus* germinated most readily on thirds (Tables 2 and 3). The germination patterns corresponded to the infection patterns.

Germination of *P. fumosoroseus* on nutrient agar (SDAY) after 12 h was 99%, and for *B. bassiana* it was 97%, using the same batch of conidia that were sprayed on the insects, so we know that viability of the spores was high.

3.3. Effect of cuticular lipids on conidial germination

B. bassiana and *P. fumosoroseus* conidia both were affected by the concentration of the whitefly cuticular extract (Fig. 2). Percent germination on the membranes

Table 1
Probit model parameters for mortality of *Bemisia argentifolii* immatures when treated with different rates of *Beauveria bassiana* and *Paecilomyces fumosoroseus* conidia (conidia/mm^2)

Pathogen	Whitefly stage	Dose–response slope (95% CI)	y-intercept (95% CI)	Pearson[a] X^2/d.f.
B. bassiana	Second instar	1.35 (1.16–1.52)	−3.70 (−3.99 to −3.42)a[b]	0.968
	Third instar	1.35 (1.16–1.52)	−3.84 (−4.16 to −3.51)b	0.968
	Fourth instar	1.35 (1.16–1.52)	−4.57 (−5.12 to −4.02)c	0.968
P. fumosoroseus	Second instar	1.17 (0.98–1.36)	−3.80 (−4.14 to −3.45)AC	0.943
	Third instar	1.17 (0.98–1.36)	−3.57 (−3.89 to −3.25)B	0.943
	Fourth instar	1.17 (0.98–1.36)	−4.27 (−4.85 to −3.69)C	0.943

[a] Scaled Pearson X^2/d.f. is an estimate of the degree of dispersion in the model. The value is expected to be near one for a well fit model.

[b] Numbers in the column followed by the same small letter or the same capital letter are not significantly different ($P \leqslant 0.05$). Comparisons of the different instars treated with *B. bassiana* are indicated with a small letter, and capital letters indicate *P. fumosoroseus* comparisons.

Table 2
Germination of conidia on whitefly cuticle after 24 h, for different life stages of *Bemisia argentifolii*, and the log odds ratios for *Beauveria bassiana* vs. *Paecilomyces fumosoroseus*

Whitefly stage	% Germination		Odds ratio[a]	95% Wald confidence limits
	B. bassiana	*P. fumosoroseus*		
Egg	13.0a[b]	7.0a	1.98	0.98–4.05
Second instar	54.0b	27.3ac	3.12*	1.93–5.05
Third instar	19.3a	45.2b	0.29*	0.17–0.49
Fourth instar	7.3a	32.7c	0.16*	0.08–0.33

[a] Odds ratios are for comparisons between fungi for a given insect stage. An asterisk indicates that the germination rates are significantly different (i.e., the odds ratio is significantly different than one, Bonferroni $\alpha \leqslant 0.05$).

[b] Numbers in a column followed by the same letter are not significantly different (Bonferroni $\alpha \leqslant 0.05$), based on their odds ratio. For the odds ratio values, see Table 3.

Table 3
Log odds ratios comparing instars for germination (after 24 h) of *Beauveria bassiana* and *Paecilomyces fumosoroseus* conidia on *Bemisia argentifolii* cuticle

Stages compared	*B. bassiana*		*P. fumosoroseus*	
	Odds ratio	95% Wald confidence limits	Odds ratio	95% Wald confidence limits
Seconds vs. eggs	7.88*	4.50–13.80	5.02	2.62–9.63
Seconds vs. fourth	14.83*	7.42–29.65	0.78	0.47–1.27
Thirds vs. eggs	1.61	0.87–2.97	11.03*	5.89–20.65
Thirds vs. second	4.90*	2.92–8.21	2.20*	1.36–3.54
Thirds vs. fourth	3.03	1.45–6.32	1.70*	1.07–2.71
Fourths vs. eggs	0.53	0.25–1.14	6.48*	3.41–12.30

[*] Odds ratios that are significantly greater than one (Bonferroni $\alpha \leqslant 0.05$).

was always greater for *P. fumosoroseus* than for *B. bassiana* in both water and yeast extract solution (Fig. 2). Germination of *B. bassiana* conidia was very poor when incubated on the membranes in water, and the logistic regression was not significant (Fig. 2). When yeast extract was added, lipid concentrations had a significant, negative effect on *B. bassiana* spore germination ($\text{logit}(y) = -0.129 - 0.42x$; slope SE = 0.07, $X^2 = 41.2$, d.f. = 1, $P \leqslant 0.0001$) (Fig. 2). Incubation medium also significantly affected *P. fumosoroseus* germination pattern ($X^2 = 129.9$, d.f. = 1, $P \leqslant 0.0001$). In the presence of

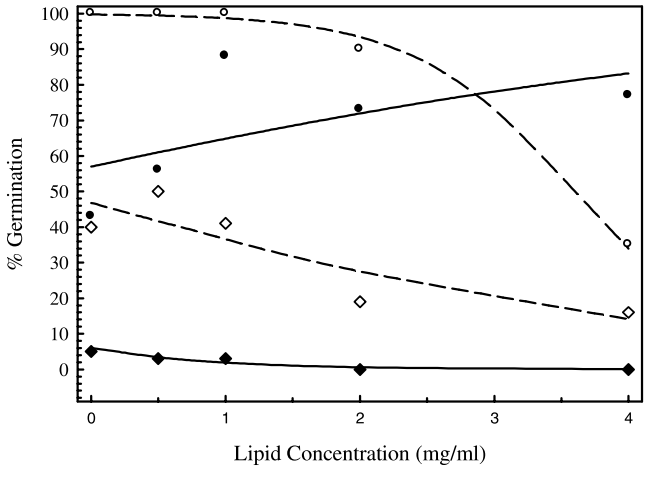

Fig. 2. Effect of cuticular lipids on percent conidial germination, after 12 h incubation at 25 °C, for *Beauveria bassiana* (diamonds) and *Paecilomyces fumosoroseus* (circles). Conidia were incubated in either water (solid symbols, solid lines) or yeast extract (open symbols, dotted lines) on membranes treated with different concentrations of lipid extract from *Bemisia bassiana* nymphs. Lines were fit using logistic regression.

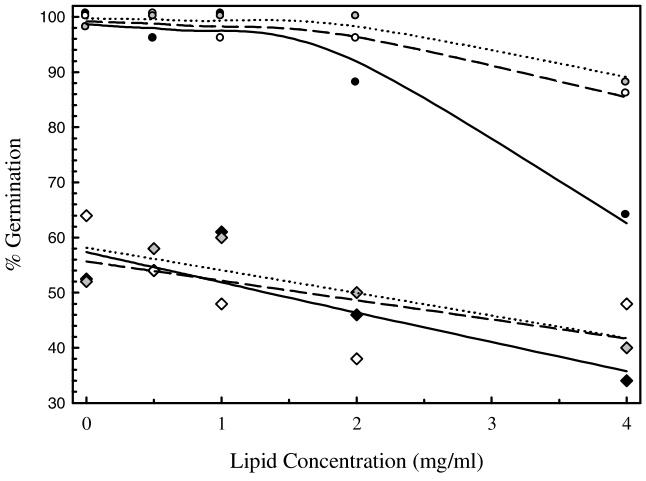

Fig. 3. Effect of cuticular lipids and synthetic long-chain fatty acids on percent conidial germination for *Beauveria bassiana* (diamonds) and *P. fumosoroseus* (circles), after 12 h incubation at 25 °C. Conidia were incubated in yeast extract on membranes treated with different concentrations of cuticular lipid (black symbols, solid lines), or the synthetic wax esters, 4 docosanyl eicosanoate (gray symbols, dashed lines), and triacontanyl tetracosanoate (open symbols, dotted lines). Lines were fit using logistic regressions.

yeast extract, *P. fumosoroseus* germination was very high, but then declined when lipid concentrations reached 2 mg/ml or higher ($\text{logit}(y) = 5.99 - 1.66x$; slope SE = 1.38, $X^2 = 145.71$, d.f. = 1, $P \leqslant 0.0001$) (Fig. 2). *P. fumosoroseus* had an unexpected response in the absence of yeast extract in that the presence of cuticular lipids increased germination rates ($\text{logit}(y) = 0.28 + 0.33x$, slope SE = 0.06) (Fig. 2), and this effect was significant ($X^2 = 27.66$, d.f. = 1, $P \leqslant 0.0001$). In the presence of yeast extract, the slope of the response curve was more negative for *P. fumosoroseus* than for *B. bassiana* ($X^2 = 12.94$, d.f. = 1, $P \leqslant 0.0001$), showing a more negative response from *P. fumosoroseus*.

3.4. Effect of long chain wax esters on spore germination

For *B. bassiana*, the effect of lipid concentration on conidial germination was only significant for the cuticular lipid ($\text{logit}(y) = 0.30 - 0.22x$; slope SE = 0.09, $X^2 = 5.7$, $P \leqslant 0.017$), and not for the synthetic lipids (Fig. 3). For *P. fumosoroseus*, increasing the lipid concentration caused a significant decline in conidial germination for the cuticular extract ($\text{logit}(y) = 4.36 - 0.96x$; slope SE = 0.17, $X^2 = 30.9$, $P \leqslant 0.0001$), and the synthetic lipids C42 ($\text{logit}(y) = 4.79 - 0.76x$; slope SE = 0.23, $X^2 = 10.72$, $P \leqslant 0.001$) and C54 ($\text{logit}(y) = 5.96 - 0.96x$; slope SE = 0.34, $X^2 = 7.99$, $P \leqslant 0.005$), but there was no significant difference in the regression lines (Fig. 3). Germination of *P. fumosoroseus* on the membranes was always greater than for *B. bassiana*, regardless of the treatment.

4. Discussion

Both *B. bassiana* and *P. fumosoroseus* were found to have similar levels of virulence to the silverleaf whitefly, but instars differed in susceptibility. Second and third instars were the most susceptible stage to *B. bassiana*, and second instars were the most susceptible to *P. fumosoroseus*. We did not test the susceptibility of eggs, but in previous studies, we found other strains of these fungi to be avirulent to eggs (R. James, unpublished data). Lacey et al. (1999) found low mortality in *B. argentifolii* eggs treated with a different strain of *P. fumosoroseus*. Also, no pathogens have been reported from field collected eggs, despite large scale efforts to find silverleaf whitefly pathogens worldwide (Lacey et al., 1996). Egg resistance to infection was consistent with the electron microscopy data we collected. First, very few conidia landed on the eggs because the eggs were laid on a leaf such that the narrowest tip faced upward, and they made a small target. Also, the conidia were covered with waxy particles from the adults, and these blow off easily, and so any conidia that land on these particles are more prone to being dislodged from the egg. In addition to few conidia being present, the proportion of conidia that germinated was very low.

The mortality response that occurred after exposure to different dosages of the fungi was lower than previously reported by us (James, 2001; James and Jaronski, 2000) and by Wraight et al. (1998), despite similar test conditions. Due to the low mortality, we were not able to accurately estimate LC_{50} levels in the probit analysis because our highest dosages only caused mean mortality levels that were around 50–70%. We tried to increase virulence of the pathogens by passing them through the host plant, but with little effect. Humidity and temperature conditions should have been optimal for infection to occur. Furthermore, Wraight et al. (2000) found that ambient humidity had little effect on infection rates in *B. argentifolii* for these fungi, probably due to microclimate effects at the leaf surface. The mostly likely explanation for differences in mortality rates is that we used a different host plant. Wraight et al. (1999), James and Jaronski (2000), and James (2001) all used cantaloupe leaves in the bioassays. Sweet potato leaves were used here because they were found to be hardier and survived the bioassay conditions for longer. Vidal et al. (1998) found that host plant had no effect on *B. argentifolii* susceptibility to *P. fumosoroseus*, however, they did not include sweet potatoes in the tests. Although our mortality levels were lower than we expected, relative comparisons between instars should still be relevant.

For both the fungi tested, conidia applied to whitefly nymphal cuticle were less likely to germinate than when they were incubated on nutrient agar. This result is consistent with what has been reported for various entomopathogenic fungi on other insects (Chandler et al., 1993; Hunt et al., 1984; Koidsumi, 1957; Madelin, 1966; Smith and Grula, 1982). However, Vega et al. (1999) observed 99% germination of *P. fumosoroseus* conidia on second and third instar silverleaf whitefly nymphs, after a similar incubation period. The difference between their results and ours is not clear.

Cuticular lipids did affect spore germination, and may account for some of the low germination rates seen on the cuticle. Thus, the waxy layer produced on the cuticle of whiteflies may act as a first line of defense against fungal pathogens. However, *P. fumosoroseus* might be less affected by these lipids because germination increased in the presence of the lipids when the incubation medium was nutrient deprived. It is possible, though not clearly shown here, that *P. fumosoroseus* is able to utilize lipids as a nutrient source when other nutrients are lacking.

Synthetic long-chain wax esters were used to test whether other long-chain fatty acids, in general, have an inhibitory effect on conidial germination. The synthetic fatty acids did inhibit conidial germination of *P. fumosoroseus*, but not to the same extent as did the cuticular lipids, and they had no effect on *B. bassiana*

germination. It is possible that the fatty acids reduced the amount of moisture available to the spores on the membranes, rather than by a direct toxic effect, but this cannot be determined for certain from our experiments (e.g., the synthetic lipids could also be toxic). Furthermore, the positive effect of cuticular lipids on *P. fumosoroseus* germination, when incubated in water with no other nutrients, may be an indication that the interaction between cuticular lipids and conidia germination is more complex than that.

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References

Abbott, W.S., 1925. A method of computing the effectiveness of an insecticide. J. Econ. Entomol. 18, 265–267.

Buckner, J.S., Hagen, M.M., Nelson, D.R., 1999. The composition of the cuticular lipids from nymphs exuviae of the silverleaf whitefly, *Bemisia argentifolii*. Comp. Biochem. Physiol. B 124, 201–207.

Chandler, D., Heale, J.B., Gillespie, A.T., 1993. Germination of the entomopathogenic fungus *Verticillium lecanii* on scales of the glasshouse whitefly *Trialeurodes vaporariorum*. Biocontr. Sci. Technol. 3, 161–167.

Faria, M., Wraight, S.P., 2001. Biological control of *Bemisia tabaci* with fungi. Crop Protect. 20, 767–778.

Hunt, D.W.A., Borden, J.H., Rahe, J.E., Whittington, R., 1984. Nutrient-mediated germination of *Beauveria bassiana* conidia on the integument of the bark beetles *Dendroctonus ponderosae* (Coleoptera: Scolytidae). J. Invert. Pathol. 44, 304–314.

James, R.R., 2001. Effects of exogenous nutrients on conidial germination and virulence against the silverleaf whitefly for two hyphomycetes. J. Invert. Pathol. 77, 99–107.

James, R.R., 2003. Combining azadirachtin and *Paecilomyces fumosoroseus* (Deuteromycotina: Hyphomycetes) to control *Bemisia argentifolii* (Homoptera: Aleyrodidae). J. Econ. Entomol. 96, 25–30

James, R.R., Jaronski, S., 2000. Effect of low viability on infectivity of *Beauveria bassiana* conidia toward the silverleaf whitefly. J. Invert. Pathol. 76, 227–228.

Koidsumi, K., 1957. Antifungal action of cuticular lipids in insects. J. Insect Physiol. 1, 40–51.

Lacey, L.A., Kirk, A.A., Millar, L., Mercadier, G., Vidal, C., 1999. Ovicidal and larvicidal activity of conidia and blastospores of *Paecilomyces fumosoroseus* (Deutermycotina: Hyphomycetes) against *Bemisia argentifolii* (Homoptera: Aleyrodidae). Biocontr. Sci. Technol. 9, 9–18.

Lacey, L.A., Fransen, J.J., Carruthers, R., 1996. Global distribution of naturally occurring fungi of *Bemisia*, their biologies and use as biological control agents. In: Gerling, D., Mayer, R.T. (Eds.), Bemisia 1995: Taxonomy, Biology, Damage Control and Management. Intercept, Ltd, Andover, pp. 401–433.

Madelin, M.F., 1966. Fungal parasites of insects. Annu. Rev. Entomol. 11, 423–448.

Pedersen, T.A., 1970. Effect of fatty acids and methyl octanoate on resting mycelium of *Boletus variegates*. Physiol. Plant 23, 654–666.

Rolinson, G.N., 1954. The effect of saturated fatty acids on oxygen uptake by *Penicillium chrysogenum*. J. Appl. Bacteriol. 17, 190–195.

Saito, T., Aoki, J., 1983. Toxicity of free fatty acids on the larval surfaces of two lepidopterous insects towards *Beauveria bassiana* (Bals.) Vuill. and *Paecilomyces fumoso-roseus* (Wize) Brown et Smith (Deuteromycetes: Moniliales). Appl. Entomol. Zool. 18, 225–233.

SAS., 1999. SAS Online Documentation. SAS Institute, Inc., Cary, NC.

Smith, R.J., Grula, E.A., 1982. Toxic components on the larval surface of the corn earworm (*Heliothis zea*) and their effects on germination and growth of *Beauveria bassiana*. J. Invert. Pathol. 39, 222–230.

Teh, J.S., 1974. Toxicity of short-chain fatty acids and alcohols towards *Cladosporium resinae*. Appl. Microbiol. 28, 840–844.

Vega, F.E., Jackson, M.A., McGuire, M.R., 1999. Germination of conidia and blastospores of *Paecilomyces fumosoroseus* on the cuticle of the silverleaf whitefly, *Bemisia argentifolii*. Mycopathology 147, 33–35.

Vidal, C., Osborne, L.S., Lacey, L.A., Fargues, J., 1998. Effect of host plant on the potential of *Paecilomyces fumosoroseus* (Deuteromycotina: Hyphomycetes) for controlling the silverleaf whitefly, *Bemisia argentifolii* (Homoptera: Aleyrodidae) in greenhouses. Biol. Control 12, 191–199.

Wraight, S.P., Carruthers, R.I., Bradley, C.A., Jaronski, S.T., Lacey, L.A., Wood, P., Galaini-Wraight, S., 1998. Pathogenicity of the entomopathogenic fungi *Paecilomyces* spp. and *Beauveria bassiana* against the silverleaf whitefly, *Bemisia argentifolii*. J. Invert. Pathol. 71, 217–226.

Wraight, S.P., Carruthers, R.I., Jaronski, S.T., Bradley, C.A., Garza, C.J., Galaini-Wraight, S., 2000. Evaluation of the entomopathogenic fungi *Beauveria bassiana* and *Paecilomyces fumosoroseus* for microbial control of the silverleaf whitefly, *Bemisia argentifolii*. Biol. Contr. 17, 203–217.